Cell
Leading Edge
Review

Life at the Leading Edge

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Cell migration requires sustained forward movement of the plasma membrane at the cell's front or "leading edge." To date, researchers have uncovered four distinct ways of extending the membrane at the leading edge. In lamellipodia and filopodia, actin polymerization directly pushes the plasma membrane forward, whereas in invadopodia, actin polymerization couples with the extracellular delivery of matrix-degrading metalloproteases to clear a path for cells through the extracellular matrix. Membrane blebs drive the plasma membrane forward using a combination of actomyosin-based contractility and reversible detachment of the membrane from the cortical actin cytoskeleton. Each protrusion type requires the coordination of a wide spectrum of signaling molecules and regulators of cytoskeletal dynamics. In addition, these different protrusion methods likely act in concert to move cells through complex environments in vivo.

Introduction

To reach their site of action, cells in multicellular animals not only move through the extracellular matrix but also on top of each other, between each other, and even through each other. For example, leukocytes attach to and migrate on endothelial cells lining the bloodstream before crossing the endothelium either between two endothelial cells or by inducing the formation of a membrane channel through a single endothelial cell (Carman, 2009). Cell migration has been studied at many different stages of animal development in vivo, as well as in numerous types of cells cultured in vitro. In order to move, cells must extend their plasma membrane forward at the front, or leading edge, of the cell. This is closely coordinated with movement of the cell body (Ridley et al., 2003).

Cells extend four different plasma membrane protrusions at the leading edge: lamellipodia, filopodia, blebs, and invadopodia. Each of these structures uniquely contributes to migration depending on the specific circumstances. For example, lamellipodia can extend long distances through the extracellular matrix in vivo, pulling cells through the tissues (Friedl and Gilmour, 2009). Filopodia explore the cell's surroundings and are particularly important for guidance of neuronal growth cones and angiogenic blood vessels (Eilken and Adams, 2010; Gupton and Gertler, 2007). Membrane blebbing has been described to drive directional cell migration during development (Charras and Paluch, 2008), and invadopodia are protrusions that allow focal degradation of the extracellular matrix, probably to facilitate invasion through the tissues (Buccione et al., 2009). These different types of protrusion can coexist at the leading edge; for example, lamellipodia, filopodia, and blebs have all been observed at the front of migrating zebrafish cells during gastrulation (Diz-Munoz et al., 2010).

Many different molecules and signaling pathways coordinate cell migration, but the actin cytoskeleton and regulators of actin dynamics are involved in all protrusions. Each actin regulator, in turn, is controlled by several signaling molecules, usually including a Rho GTPase, membrane phospholipids, and protein phosphorylation.

Rho GTPases are critical signal transducers that transmit signals from membrane receptors to the cytoskeleton and cell adhesions. Most Rho GTPases switch between an active GTP-bound conformation, which interacts with downstream effectors, and an inactive GDP-bound conformation. GTP hydrolysis converts Rho GTPases from the active to inactive form. Although they have intrinsic GTPase activity, their hydrolysis rates are normally slow and are accelerated in cells by GTPase-activating proteins (GAPs). Exchange of GDP for GTP induces activation, and this is catalyzed by guanine nucleotide exchange factors (GEFs). There are two families of GEFs: DH-PH domain-containing Dbl-related GEFs and DHR2 domain-containing DOCK family GEFs (Buchsbaum, 2007). Rho GTPases interact with membranes through, at least in part, lipid groups covalently attached posttranslationally, including farnesyl and geranylgeranyl isoprenoids. Some Rho GTPases are also regulated by Rho GDP-dissociation inhibitors (RhoGDIs); these proteins bind to isoprenoids and, hence, solubilize and extract the Rho GTPases from membranes (Buchsbaum, 2007).

This Review discusses how actin regulators contribute to the formation of the four protrusion types currently known to occur at the leading edge of migrating cells: lamellipodia, filopodia, blebs, and invadopodia. The Review describes the signaling molecules that activate these actin regulators and thus allow cells to respond dynamically to their extracellular environment with the most appropriate type of protrusion.

Lamellipodia

Actin Regulators

The thin sheet-like region at the leading edge of migrating fibroblasts in culture was first named a "lamellipodium" by Michael

Abercrombie in 1970 (Abercrombie et al., 1970). In elegant electron microscopy studies, he and colleagues showed that lamellipodia contain microfilaments (i.e., actin filaments) but not microtubules (Abercrombie et al., 1971). We now know that actin polymerization drives forward protrusion of the plasma membrane in lamellipodia (Ridley et al., 2003). Behind the highly dynamic lamellipodium is a more stable region, called the lamella, which contributes to cell migration by coupling the actin network to myosin II-mediated contractility and substrate adhesion (Ponti et al., 2004). Lamellipodia are observed in many different cell types moving in vivo, such as muscle precursors in chick embryos, epithelial and follicular epithelium border cells in *Drosophila*, and neural crest cells in *Xenopus* and zebrafish (Friedl and Gilmour, 2009; Weijer, 2009).

For many years, the Arp2/3 complex was thought to be the primary mediator of actin polymerization in lamellipodia. First discovered to nucleate actin polymerization in 1998 (Mullins et al., 1998), the Arp2/3 complex binds to the sides of actin filaments and stimulates the formation of branched ''dendritic'' actin filament networks (Campellone and Welch, 2010) (Figure 1). The Arp2/3 complex remains associated with filament pointed ends, and it is distributed throughout the lamellipodium, but it is incorporated only into the network at the front of the lamellipodium (Lai et al., 2008). In vitro, the nucleation-promoting factors of the WASP (Wiskott–Aldrich syndrome protein) family stimulate the ability of the Arp2/3 complex to induce actin polymerization. These factors, which include WASP itself, N-WASP, WAVE1–3, WASH, and WHAMM proteins, all bind to the Arp2/3 complex through a C-terminal acidic domain. WAVE proteins are known to localize to the leading edge and contribute to lamellipodium extension, whereas N-WASP may localize to and affect lamellipodia in some cell types or indirectly through its role in endocytosis (Campellone and Welch, 2010). WAVE1–3 exist in stable pentameric complexes with Abelson Interacting Protein (Abi), PIR121 (also known as Sra or CYFIP), Nck Associated Protein 1 (Nap1), and HSPC300 (Derivery and Gautreau, 2010). WASH is part of a similar complex that regulates actin polymerization on endosomes (Rottner et al., 2010), and N-WASP can also bind to Abi, but in this case the Abi1/N-WASP complex regulates endocytosis (Takenawa and Suetsugu, 2007).

More recently, other actin nucleators have been found to contribute to lamellipodium extension, including several members of the formin and Spire families. Formins protect barbed (+) ends of actin filaments from capping, and they promote filament elongation without branching (Figure 1). The formin mDia1, a Rho target, localizes at the leading edge (Chesarone et al., 2010). Members of the Spire family have multiple WH2 domains, which bind actin monomers and nucleate unbranched actin filaments. One of these proteins, Cordon-Bleu, is localized in lamellipodia and, when overexpressed, it increases the number of cells with lamellipodia and membrane ruffles (Campellone and Welch, 2010). JMY (junction-mediating and regulatory protein) is an unusual actin nucleator in that it has three WH2 domains and thus can nucleate unbranched actin filaments, like Spire, in the absence of the Arp2/3 complex. In addition, JMY has an acidic domain that binds to and stimulates the Arp2/3 complex and hence induces branched filaments (Zuchero et al., 2009). Although JMY is often in the nucleus, it also localizes at the leading edge, particularly in rapidly migrating neutrophils, and it regulates migration into scratch wounds. It would be interesting to determine whether JMY uses both of its actin nucleation activities at the leading edge to contribute to protrusion.

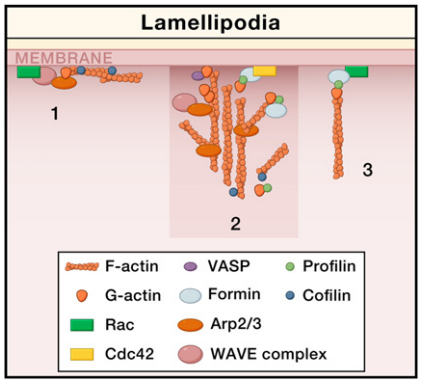

Figure 1. Lamellipodia

A model for lamellipodium formation is as follows: (1) Severing of actin filaments by cofilin provides free actin filament barbed ends, which act as sites for actin polymerization and subsequently Arp2/3-mediated nucleation of new filaments. (2) In conditions of steady-state lamellipodial extension, actin polymerization in lamellipodia is nucleated by the Arp2/3 complex, generating a branched actin filament network. The Arp2/3 complex is activated by the WAVE complex, which in turn is activated at the membrane by Rac1. Formins extend Arp2/3 complex-generated filaments. Formins are activated by Cdc42, Rac1, and probably other Rho GTPases. Actin monomers (G-actin) are provided to formins by profilin. VASP also contributes to actin filament extension. Cofilin severs and depolymerizes older actin filaments in the network. (3) Formins can also nucleate actin filaments independent of the Arp2/3 complex, generating unbranched filaments.

Branched actin filament networks have been found in electron microscopy images of lamellipodia (Svitkina and Borisy, 1999). However, the extent of actin filament branching in lamellipodia may vary depending on the cell type and conditions of fixation because a recent report found only few filament branches near the leading edge of cells (Urban et al., 2010). It could be that the balance of actin nucleation by the Arp2/3 complex relative to formins and Spires is key to how branched the network is at the leading edge. Indeed, the formin mDia2 is involved in generating long actin filaments in lamellipodia (Yang et al., 2007). In addition, different kinds of actin nucleators can work synergistically to promote actin polymerization (Chesarone and Goode, 2009). For example, mDia1, N-WASP, and WAVE2 all contribute to cell protrusion induced by epidermal growth factor (EGF) (Sarmiento et al., 2008). Adenomatosis polyposis coli (APC) has been recently described to have actin-nucleating activity and to act together with mDia1 (Okada et al., 2010). One possibility is that the Arp2/3 complex and/or Spires initiate nucleation in lamellipodia, whereas formins promote elongation.

The balance of other actin-binding proteins also contributes to the length of actin filaments in the lamellipodium. More capping protein activity reduces actin filament length and increases nucleation by the Arp2/3 complex by diverting actin monomers from elongation to nucleation (Akin and Mullins, 2008). On the other hand, more VASP, which promotes filament elongation, generates more long filaments (Bear and Gertler, 2009;

Breitsprecher et al., 2011). Cofilin mediates the severing of existing cortical actin filaments, which generates new barbed ends and hence new filaments, to which the Arp2/3 complex can then bind and stimulate branching (van Rheenen et al., 2009) (Figure 1). Cortactin is a scaffolding protein that stabilizes Arp2/3 complex-induced branches and affects lamellipodial persistence (Lai et al., 2009; Ren et al., 2009). Super-resolution imaging (Toomre and Bewersdorf, 2010) might allow effects of each actin nucleator to be determined more precisely because the technique will permit the observation of actin filament arrangements in lamellipodia by optical microscopy, which requires less harsh fixation conditions than those required for electron microscopy.

There is also strong evidence that, in addition to actin polymerization, myosin II activity is required for stable lamellipodial extension, at least in cultured cells. Periodic myosin II-based contractions occur at the back of the lamellipodium (Giannone et al., 2007). These contractions could allow the protrusion to sense the pliability of the extracellular matrix and other cells and to determine the direction of migration. Myosin II activity is also implicated in actin filament disassembly at the back of the lamellipodium (Wilson et al., 2010).

Finally, several actin nucleators interact directly with microtubules, including the mDia proteins, APC, Spire, and WHAMM (WAS protein homolog associated with actin, golgi membranes, and microtubules). WHAMM's ability to bind microtubules probably relates to its functions in Golgi transport. Spire was reported to localize with Rab11 on endosomes and the Golgi, but it is not known whether it functions to nucleate actin filaments with formins at these sites or interacts with microtubules on a trafficking route to the plasma membrane (Campellone and Welch, 2010). The mDia proteins stabilize microtubules (Chesarone et al., 2010), whereas APC contributes to cell migration by capturing and stabilizing microtubule tips in the lamellipodium (Etienne-Manneville, 2009). It therefore seems likely that mDia and APC could coordinately regulate actin and microtubule cytoskeletons at the leading edge.

Signaling Molecules

Many extracellular stimuli induce the formation of lamellipodia, including growth factors, cytokines, and cell adhesion receptors; a myriad of signaling and structural proteins have been implicated in this process over the past 20 years. Rho GTPases act coordinately with other signals to activate actin regulators in lamellipodia (Figure 1).

Using biosensors, active Rac1, RhoA, and Cdc42 have been shown to localize in lamellipodia during protrusion (Machacek et al., 2009). Activation of Rac1 by itself, using a photoactivatable Rac1, is sufficient to induce lamellipodium extension (Wu et al., 2009), and it would be interesting to know whether this involves RhoA and Cdc42. Rho GTPases can be activated by multiple different GEFs at the leading edge, depending on the cell type and extracellular stimulus (Buchsbaum, 2007). More complex signaling is achieved through activation of GEFs by other Rho GTPases. For example, RhoG activates Rac/Cdc42 through its target protein ELMO (EnguLfment and cell MOtility) and DOCK family GEFs (Cote and Vuori, 2007). RhoG can also induce lamellipodia through an unknown Rac-independent pathway (Meller et al., 2008). Regulated localization of Rho GTPases is also important for their function: Rac is known to be recruited to the plasma membrane at the leading edge through vesicle trafficking (Donaldson et al., 2009), and multiple phosphorylations alter RhoGDI binding to Rho GTPases (Harding and Theodorescu, 2010).

Rac activates the pentameric WAVE complex, but it is currently unknown whether there is any difference in the ability of the three Rac isoforms, Rac1, Rac2, and Rac3, to interact with the complex. Rac binds to PIR121 in the WAVE complex (Takenawa and Suetsugu, 2007). Structural analysis of the WAVE complex indicates that the C-terminal WCA domain of WAVE, which activates the Arp2/3 complex, is normally sequestered within the complex. It is predicted that Rac binding would induce structural rearrangements to allow the WCA domain to become accessible on the surface (Chen et al., 2010). The Rac target IRSp53 (insulin receptor tyrosine kinase substrate p53) contributes to lamellipodium extension by binding to Rac and WAVE2. Interestingly, the role of IRSp53 in lamellipodia can be selectively inhibited by Kank, an ankyrin repeat-containing protein that inhibits the binding of Rac but not Cdc42 to IRSp53 (Roy et al., 2009). WAVEs are also activated by tyrosine and serine/threonine phosphorylation, and again these phosphorylations are predicted to alter WAVE complex structure. Indeed, phosphomimicking mutations activate actin polymerization and lamellipodium formation (Chen et al., 2010; Sossey-Alaoui et al., 2007). Phosphorylation of Arp2, a component of the Arp2/3 complex, is important for its association with the pointed ends of actin filaments, which is required for it to induce effective filament branching and hence contribute to lamellipodium formation (LeClaire et al., 2008). Finally, the Rac target PAK (p21-activated protein kinase) may be involved in regulating the delivery of WAVE2 to the plasma membrane (Takahashi and Suzuki, 2009).

Cofilin/ADF is inhibited by phosphorylation, by binding to phosphatidylinositol 4,5-bisphosphate, and by increased pH (van Rheenen et al., 2009). Cdc42 and Rac act through their targets PAK and LIMK to phosphorylate and decrease the activity of cofilin (Bernard, 2007), which is probably important to allow cofilin recycling back to the membrane to generate new barbed ends for actin polymerization (van Rheenen et al., 2009) and to regulate the width of the lamellipodium (Delorme et al., 2007). This function of Rac could explain why Rac is most active slightly further back in the lamellipodium than RhoA (Machacek et al., 2009), although RhoA/ROCK can also phosphorylate and inhibit cofilin/ADF (Bernard, 2007). Indeed, both PAK and ROCK appear to regulate cofilin phosphorylation at the leading edge (Delorme et al., 2007). The Rac target NADPH oxidase, which generates reactive oxygen species (ROS), has also been implicated in lamellipodia (Nimnual et al., 2003). One possible mechanism whereby ROS could contribute to lamellipodia is through cofilin; ROS lead to cofilin dephosphorylation through activation of the cofilin phosphatase Slingshot (Kim et al., 2009).

RhoA has predominantly been implicated in tail retraction of migration cells (Ridley et al., 2003), but it is clearly active at the front of lamellipodia where it might act to stimulate mDia1-mediated actin polymerization and/or myosin II-mediated retraction events (Pertz, 2010). Notably, RhoA is also highly active in membrane ruffles that retract backward from lamellipodia (Pertz

et al., 2006). The RhoGEF GEF-H1 could be important for activating RhoA in lamellipodia because GEF-H1 knockdown decreases RhoA activation at the leading edge and reduces forward protrusion (Nalbant et al., 2009). Although RhoA depletion induces loss of lamellipodia (Heasman et al., 2010), too much RhoA activity at the front inhibits lamellipodial extension. RhoA levels have been reported to be regulated locally in lamellipodia by Smurf1- (SMAD-specific E3 ubiquitin protein ligase) and ubiquitin-mediated degradation. Moreover, decreasing the expression of Smurf1 reduces lamellipodia and increases membrane blebbing (Sahai et al., 2007; Wang et al., 2003). However, interpretation of these results is complicated by the fact that Smurf1 has multiple targets, several of which affect cell migration (Huang, 2010).

Lamellipodial extension is abruptly terminated by contact inhibition, when two cells of the same type touch. Michael Abercrombie first described contact inhibition between migrating fibroblasts (Abercrombie and Heaysman, 1954), and studies more recently have explored the mechanisms underlying contact inhibition. For example, ephrin (Eph) receptors inhibit lamellipodial extension by activating myosin II-mediated retraction through a combination of Cdc42/MRCK and RhoA/ROCK signaling (Astin et al., 2010; Groeger and Nobes, 2007). In vivo, contact inhibition is used during development to guide the migration of neural crest cells. Noncanonical Wnt signaling leads to activation of RhoA at sites of contact between neural crest cells, which then represses lamellipodia through its target ROCK (Carmona-Fontaine et al., 2008), presumably through increased actomyosin contractility. It is interesting that RhoA can either contribute to or inhibit lamellipodial extension depending on the circumstances, which might reflect involvement of different RhoGEFs and downstream targets (Heasman et al., 2010).

Filopodia

Actin Regulators

Filopodia are exploratory extensions from the plasma membrane that contain parallel bundles of actin filaments (Figure 2). Fascin is the major actin-bundling protein that localizes to filopodia and is important for filopodium stability (Machesky and Li, 2010). One model for filopodia assembly is that they emerge from the lamellipodial F-actin network nucleated by the Arp2/3 complex through the binding of proteins such as fascin and the anti-capping protein VASP (Vasodilator-stimulated phosphoprotein) (Gupton and Gertler, 2007). Indeed, N-WASP is required for filopodium formation in certain situations, and the Arp2/3 complex can be active in filopodia (Johnston et al., 2008; Takenawa and Suetsugu, 2007). However, filopodia can also be observed independent of N-WASP, the Arp2/3 complex, and lamellipodia (Takenawa and Suetsugu, 2007), and it is now clear that formins, in particular the mDia proteins, are major contributors to actin polymerization in filopodia (Mellor, 2010; Campellone and Welch, 2010).

VASP and its relatives Mena and Evl (known as Ena/VASP proteins) localize to tips of filopodia, and at least in certain systems, they are essential for filopodium extension (Bear and Gertler, 2009). In vitro, Ena/VASP proteins have an anti-capping protein function, and they promote filament elongation (Hansen and Mullins, 2010). VASP oligomers could stimulate filament elongation in filopodia by delivering actin monomers to the growing tips of filopodia (Applewhite et al., 2007; Breitsprecher et al., 2008) but also by inhibiting filament capping (Bear and Gertler, 2009).

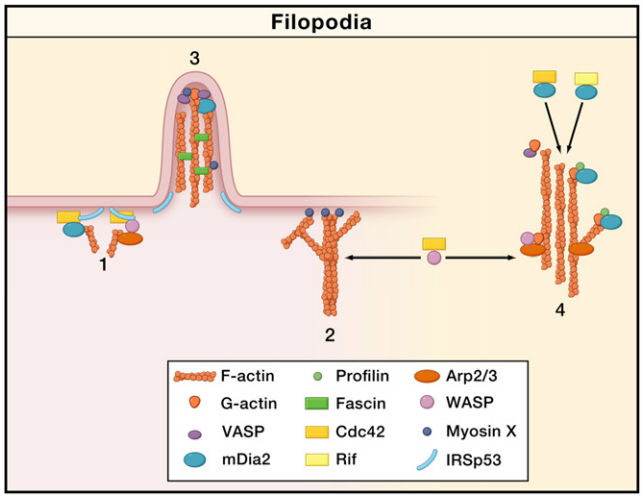

Figure 2. Filopodia

A model for filopodium formation is as follows: (1) IRSp53 initiates filopodia by bending the membrane and recruiting Cdc42 and Cdc42 targets, mDia2 and WASP/N-WASP, which then stimulate actin polymerization. (2) Actin filaments could also be provided from lamellipodia, where Myosin X could cluster WASP/Arp2/3-nucleated actin filaments. (3) Filopodia subsequently extend through the addition of actin monomers (G-actin) onto actin filaments (F-actin). VASP, Myosin X, and mDia2 are localized to the tip of filopodia. Myosin X moves dynamically in filopodia and could contribute to delivery of proteins to the filopodial tip. (4) Actin polymerization in filopodia is nucleated by mDia2 in concert with VASP, which delivers actin monomers to the filopodial tip. Profilin binds to and provides actin monomers directly to mDia2. Cdc42 and Rif stimulate mDia2-mediated actin polymerization, and Cdc42 also stimulates WASP/Arp2/3-driven polymerization.

Overexpression of a variety of proteins can increase the number of filopodia on cells. For example, filopodia can be induced by proteins containing I-BAR domains, which bend the plasma membrane outwards (Figure 2). The IRSp53 protein is a multidomain protein that induces filopodia through its I-BAR domain. The I-BAR domain alone induces small dynamic filopodium-like membrane protrusions lacking F-actin. IRSp53 also interacts with N-WASP, which is required for IRSp53-induced filopodium formation, even though in other conditions N-WASP is not required for filopodium formation (Ahmed et al., 2010; Takenawa and Suetsugu, 2007).

Myosin X traffics to the tip of filopodia and can induce filopodium assembly (Sousa and Cheney, 2005). The selectivity of myosin X for filopodia appears to be due to its preferential movement on fascin-actin bundles (Nagy and Rock, 2010). Two mechanisms have been proposed to explain how myosin X stimulates filopodium assembly (Figure 2). First, it could deliver cargo, such as actin monomers, to the growing tips of filopodia and, hence, accelerate filament elongation (Zhuravlev et al., 2010). Second, its motor function could induce actin filament convergence at the leading edge to initiate filopodium extension (Tokuo et al., 2007).

Signaling Molecules

Cdc42 was the first Rho GTPase found to induce filopodia. Cdc42 could bring together three of its targets, IRSp53 (I-BAR protein), mDia2, and N-WASP, all of which can contribute to filopodium initiation and extension (Ahmed et al., 2010) (Figure 2). I-BAR domains may activate membrane protrusion by clustering membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) (Zhao et al., 2011), which could then contribute to activation of PIP_2-binding proteins, such as N-WASP. N-WASP is also activated by tyrosine phosphorylation (Takenawa and Suetsugu, 2007), and thus, it is regulated cooperatively by multiple signals (Figure 2). In contrast, IRSp53 function is inhibited by threonine phosphorylation and subsequent binding to 14-3-3 proteins (Robens et al., 2010). In addition to Cdc42, other Rho GTPases can also induce filopodia (Mellor, 2010). For example, RhoF/Rif induces filopodia via mDia2, and this is important in the early stages of dendritic spine assembly in neurons (Hotulainen et al., 2009). Whether RhoF also recruits I-BAR proteins remains to be determined.

Fascin and Ena/VASP binding to actin filaments is inhibited by phosphorylation (Bear and Gertler, 2009; Machesky and Li, 2010). Protein kinase C (PKC) phosphorylates fascin, and Rac regulates the interaction of fascin with PKC (Parsons and Adams, 2008), and thus, Rac might inhibit filopodium assembly. Alternatively, it is possible that fascin in association with PKC has a separate function independent of its actin-bundling activity (Hashimoto et al., 2007). Interestingly, Rab35 interacts directly with fascin and could be important for its delivery to filopodia (Zhang et al., 2009). Rab35 induces long filopodium-like protrusions and is required in *Drosophila* cells for Cdc42 delivery to the plasma membrane (Chua et al., 2010; Shim et al., 2010). It will be interesting to know whether Cdc42 and fascin delivery is coordinated by Rab35 to ensure that they act together in stimulating filopodium extension.

How is filopodium extension terminated? Formin displacers or inhibitors could be important in this process. In budding yeast, BUD14 (Bud site selection protein 14) directly binds to the FH2 domain of the Bnr formin and displaces it from the barbed end of actin filaments (Chesarone et al., 2009). It is not yet known whether mammalian cells have a protein that acts similarly to BUD14. In mammalian cells, overexpression of Dia-interacting protein (DIP) inhibits mDia2-induced actin assembly in vitro and filopodium formation in vivo (Eisenmann et al., 2007).

Invadopodia

Actin Regulators

Invadopodia were first described as actin-rich matrix-degrading protrusions in Rous sarcoma virus-transformed fibroblasts, driven by oncogenic Src tyrosine kinase (Chen, 1989). Invadopodia and related structures known as podosomes are important for degrading the extracellular matrix during cell invasion (Buccione et al., 2009), particularly when cells cross the basement membrane (Schoumacher et al., 2011). Invadopodium extension in three dimensions (3D) requires force driven by actin polymerization. Many studies on invadopodia are carried out on two-dimensional (2D) surfaces coated with extracellular matrix proteins, where they are present on the ventral surface. In 3D, invading cells often extend long protrusions that degrade the matrix (Friedl and Gilmour, 2009; Schoumacher et al., 2010; Wolf and Friedl, 2009).

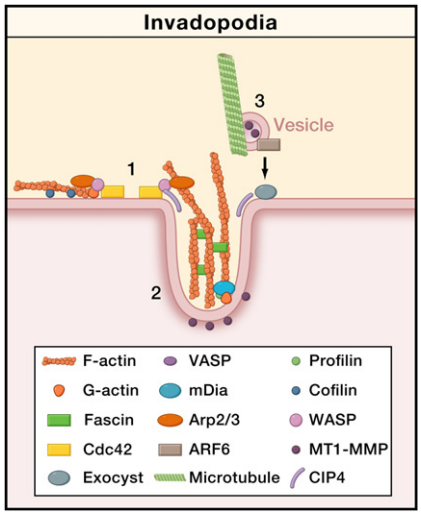

Figure 3. Invadopodia

A model for invadopodium assembly is as follows: (1) Actin polymerization in invadopodia is mediated by Cdc42-mediated N-WASP/WASP activation of the Arp2/3 complex. Cofilin severs actin filaments (F-actin) to provide new sites for actin nucleation. CIP4 might bend membranes and also help to recruit Cdc42/N-WASP. (2) Actin filament elongation in the invadopodium requires mDia formins and profilin/actin monomers (G-actin). Actin filaments are bundled by fascin. (3) MT1-MMP and possibly other MMPs are transported to the invadopodial tip by vesicle trafficking, initially on microtubules, and requiring ARF6. The exocyst captures vesicles at the plasma membrane.

Although many of the actin-regulatory proteins found in invadopodia are also in filopodia and lamellipodia, the key difference is that invadopodia degrade the extracellular matrix, and thus, they require the delivery of vesicles containing matrix-degrading proteases, particularly membrane type 1 metalloprotease (MT1-MMP). These vesicles are targeted to invadopodia by the vesicle-tethering exocyst complex (Poincloux et al., 2009) (Figure 3). Microtubules are also important for invadopodium extension, probably for the delivery of vesicles, and intermediate filaments might provide mechanical stability (Schoumacher et al., 2010).

The Arp2/3 complex is an essential component of invadopodia, and N-WASP (and WASP in hematopoietic cells) appears to be the major Arp2/3 complex activator (Buccione et al., 2009) (Figure 3). However, the formins mDia1–3 are also required for invadopodium assembly and invasion, implying that Arp2/3 complex and formins cooperate to induce actin polymerization in invadopodia (Lizarraga et al., 2009), as in lamellipodia and filopodia. Several actin-binding proteins also contribute to formation of invadopodia. For example, cortactin binds to and buffers cofilin: cortactin phosphorylation releases cofilin so that it can sever filaments to create new barbed ends for actin polymerization; then dephosphorylated cortactin inhibits cofilin's actin-severing activity to promote filament elongation (Oser et al., 2009). In addition, the actin-bundling protein fascin has recently been shown to be critical for invadopodium stability (Machesky and Li, 2010).

Signaling Molecules

Cdc42 is the main Rho GTPase implicated in the formation of invadopodia, and it appears to coordinate actin filament assembly with matrix degradation. Cdc42 is required for N-WASP/WASP targeting; it is not yet known whether Cdc42 also regulates mDia proteins in invadopodia. Cdc42 could be activated to form invadopodia by the RhoGEF Fgd1 (faciogenital dysplasia protein), which is mutated in faciogenital dysplasia (Ayala et al., 2009). Generation of membrane curvature is also important for invadopodium assembly, as for filopodia. Recently, the F-BAR-containing protein CIP4 has been implicated in invadopodia (Pichot et al., 2010). CIP4 also binds Cdc42 and N-WASP (Figure 3), thereby acting as a membrane-curving scaffolding protein similar to IRSp53 in filopodia.

A role for RhoA in invadopodia has also been suggested, but its contribution is not clear, apart from a possible role in regulating exocyst binding to IQGAP1, which can also be mediated by Cdc42 (Buccione et al., 2009).

Tyrosine kinases of the Src family stimulate invadopodium assembly, and indeed many Src substrates are in invadopodia, including cortactin and WASP/N-WASP, for which tyrosine phosphorylation is important for podosome assembly (Dovas and Cox, 2010). Reactive oxygen species generated by NADPH oxidases are required for assembly of invadopodia (Diaz et al., 2009; Weaver, 2009), and NADPH oxidase components, such as Tks, are Src substrates implicated in formation of invadopodia (Buccione et al., 2009). Abl tyrosine kinases also appear to be important for targeting or retaining MT1-MMP in invadopodia (Smith-Pearson et al., 2010).

In summary, it appears that Cdc42 and tyrosine kinases act coordinately to drive both the actin polymerization required for invadopodium extension and the delivery and retention of MT1-MMP to the surface of invadopodia (Figure 3).

Blebs

Actin Regulators

Membrane blebbing was first described in migrating amphibian and fish cells in several papers between the 1940s and the 1970s (Charras and Paluch, 2008). Recently, blebbing has received renewed interest in the migration field with the observations that multiple cell types move by blebbing under certain conditions, including cancer cells and *Dictyostelium* cells in vitro and several cell types in vivo (Charras and Paluch, 2008; Fackler and Grosse, 2008). In vitro, blebbing is often observed on or within pliable extracellular matrix environments, in contrast to the predominance of lamellipodia on rigid substrates.

Blebs form when the plasma membrane detaches focally from the underlying actin filament cortex, allowing cytoplasmic flow to push the membrane outwards rapidly due to hydrostatic pressure in the cell interior (Bovellan et al., 2010). Myosin II-induced actomyosin contraction increases hydrostatic pressure locally or globally leading to focal rupture of the actin cortex from the membrane, thereby driving the formation of blebs (Tinevez et al., 2009). Reduced association between the cortex and the membrane could also drive blebbing, for example in filamin null cells (filamin is an actin filament cross-linking protein critical for stability of the actin cortex) (Charras and Paluch, 2008).

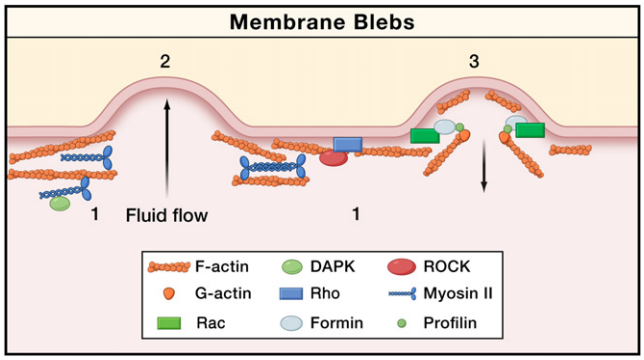

Figure 4. Membrane Blebs

A model for membrane blebbing is as follows: (1) Membrane blebs are induced by local weakening of plasma membrane/cortical actin interactions, coupled to actomyosin contractility on the membrane. Actomyosin contractility can be induced by Rho/ROCK and/or DAPK stimulation of myosin light chain phosphorylation on myosin II. (2) This leads to fluid flow pushing the membrane outwards locally. (3) Actin polymerization on the membrane in blebs leads to re-engagement of the plasma membrane with cortical actin filaments and retraction of blebs. This polymerization might be mediated by formins such as mDia proteins or FHOD1 and require activation by Rac1.

Once blebs have extended, actin filaments reassemble on the bleb membrane to form a new actin cortex (Figure 4). The actin nucleator required to stimulate actin polymerization is likely to vary depending on the cell type. Several formins have been implicated in membrane blebbing. Stabilization of the actin cortex requires ERM (Ezrin, Radixin, and Moesin) proteins, which link actin filaments to the plasma membrane and to membrane receptors. Finally, Myosin II is recruited, and actomyosin contraction can power retraction of membrane blebs. Thus, contractility not only can induce blebbing but also contribute to bleb termination (Charras and Paluch, 2008; Fackler and Grosse, 2008). In migrating cells, however, blebs are not always retracted; instead, new blebs extend out of existing blebs (Kardash et al., 2010).

Leading edge extension through blebbing and lamellipodia is not mutually exclusive. For example, both structures can be observed in different regions or at different times of the extending membrane in zebrafish prechordal plate precursor cells during gastrulation (Diz-Munoz et al., 2010). However, an increase in blebbing leads to a decrease in lamellipodia and vice versa (Derivery et al., 2008), reflecting the very different mechanical processes: blebbing requires loss of actin filament interaction with the membrane, whereas lamellipodium extension requires close interaction of actin filaments with the membrane.

Signaling Molecules

Cells bleb during the initial stages of adhesion to extracellular matrix proteins before they adhere firmly (Dubin-Thaler et al., 2008). This appears to correlate with rates of membrane endocytosis and exocytosis, suggesting that blebbing is due to excess membrane (Norman et al., 2010). Rho and its target ROCK induce blebbing through increased actomyosin contractility, but it is not known which of the three Rho isoforms (RhoA, RhoB, or RhoC) is actually responsible for blebbing during migration. Microtubule depolymerization activates RhoA/ROCK,

which then leads to membrane blebbing and bleb-based migration (Pletjushkina et al., 2001; Takesono et al., 2010). Germ cells in zebrafish extend membrane blebs at the leading edge during migration. Interestingly, both RhoA and Rac1 are active in this region (Kardash et al., 2010). Inhibition of Rho isoforms prevents membrane blebbing, whereas Rac1 is required for actin polymerization at the front. Rac1-induced actin polymerization, in turn, is dependent on E-cadherin engagement, which is well known to stimulate Rac1 (Yap and Kovacs, 2003). This suggests a model in which Rho/ROCK-induced bleb extension is followed by adhesion-stimulated Rac-driven actin polymerization, both of which are required for sustained membrane extension during directional migration in vivo.

Apart from ROCK, other kinases can also stimulate myosin light chain (MLC) phosphorylation in membrane blebbing, including the Death-associated protein kinase (DAPK) (Bovellan et al., 2010). DAPK can act redundantly with ROCK to regulate phosphorylated MLC levels (Neubueser and Hipfner, 2010), and hence ROCK-independent blebbing could involve DAPK.

Several formins are linked to membrane blebbing (Figure 4), but whether they contribute to physiological blebbing during migration is not yet known. Diaphanous-interacting protein (DIP) is a scaffold protein that induces membrane blebbing when overexpressed, presumably because it binds to and inhibits the activity of the formin mDia2 (Eisenmann et al., 2007). This suggests that mDia2 could be involved in stabilizing the actin cortex, thereby inhibiting detachment of the membrane from the cortex. Overexpression of the formin FHOD1 (FH2 domain-containing protein 1) reduces the size of ROCK1-induced membrane blebs but increases their number (Hannemann et al., 2008). This suggests that FHOD1 too might stabilize the actin cortex and/or stimulate actin polymerization in blebs to promote bleb retraction. On the other hand, overexpression of a constitutively activated form of the formin FMNL1 (Formin-like protein 1) alone induces membrane blebbing, independently of ROCK (Han et al., 2009), and thus this response might require DAPK. It will be interesting to determine whether FMNL1 stimulates blebbing by thickening the actin cortex and hence increasing cortical tension.

Collective Cell Migration

Collective migration is the simultaneous movement of multiple cells attached to each other through cell-cell adhesion, which occurs reiteratively during development and wound healing (Weijer, 2009). Live-cell imaging during development has shown that the leading cells of collectively migrating groups selectively extend lamellipodia, filopodia, and/or blebs, whereas cells behind rarely extend protrusions (Friedl and Gilmour, 2009; Diz-Munoz et al., 2010).

Recent data have indicated the involvement of Rho GTPases and other signaling pathways in collective cell migration. It is clear that Rac-driven lamellipodial extension is important in the leader cells of collective groups in a number of models. For example, during angiogenesis, the tip cells at the front of sprouting blood vessels extend long protrusions, which are presumed to be required for navigation sensing, such as guidance toward VEGF (vascular endothelial growth factor) (Eilken and Adams, 2010) . Rac1 is required for VEGF-induced endothelial sprouting, and local downregulation of myosin II on the cortex is important for tip branching in 3D (Eilken and Adams, 2010). Similarly, duct initiation in mammary epithelial morphogenesis requires Rac (Ewald et al., 2008), and *Drosophila* epithelial border cells extend long Rac-driven protrusions in order to migrate as a group between egg chamber cells toward the oocyte (Friedl and Gilmour, 2009). Rac activation in the leading cell, using a photoactivatable Rac1, is sufficient to drive polarization of the border cell cluster (Wang et al., 2010). Consistent with its role in lamellipodia (see above), the balance of Rac-regulated cofilin phosphorylation is also critical for collective border cell migration. It is possible that high levels of phospho-cofilin could suppress protrusion in all cells of the group, except the leading cell (Zhang et al., 2011). Alternatively, cofilin and its regulator LIMK could be critical for matrix degradation, which is particularly important in the leading cell of collectively migrating cancer cells (Scott et al., 2010).

Maintaining intact cell-cell adhesions is essential for collective migration. In A431 cancer cells, this maintenance has been shown to require the transmembrane receptor DDR1 (Discoidin domain receptor tyrosine kinase 1), which recruits the Par polarity complex to cell-cell junctions and reduces actomyosin contractility (Hidalgo-Carcedo et al., 2011). The Par complex, in turn, is known to be important for assembly of cell-cell junctions and is often regulated by Cdc42 (Goldstein and Macara, 2007). However, whether Cdc42 contributes to collective migration of cancer cells has not been investigated. Suppression of actomyosin contractility at cell-cell junctions also enhances endothelial vessel sprouting (Abraham et al., 2009), indicating that the balance of forces acting on cell-cell interactions is critical for collective movement of cells.

Conclusions and Perspectives

Since the "textbook" model for actin dynamics in lamellipodia was first described by Pollard and Borisy (2003), our understanding of how protrusions are initiated, extended, and retracted has increased on numerous fronts. It is now clear that multiple actin nucleators are involved in each type of protrusion and that some formins and Spire family proteins have important roles at the leading edge. Different protrusions can exist together, such as filopodia and lamellipodia at the leading edge of some cell types, whereas in other cases they act independently. For example, filopodia are initiated in the absence of lamellipodia as the starting point for dendritic spines on neurons (Yoshihara et al., 2009). Cells can also switch rapidly between different types of protrusion. For example, in *Dictyostelium*, blebs interchange rapidly with filopodia and lamellipodia at the leading edge (Yoshida and Soldati, 2006).

Multiple signals regulate protrusions, but how they act together to coordinate protrusion extension and retraction is currently not yet clear. For example, although we now know that the Rho GTPases RhoA, Rac1, and Cdc42 are all active in lamellipodia, we still do not know precisely where and when they interact with each of their downstream targets. RhoA is active in areas of membrane blebbing, but where exactly it stimulates actomyosin contraction with respect to where the bleb extends is not clear. We know even less about where Rho GTPases are active in filopodia or invadopodia. In many cases, Rho GTPases need to act synergistically with other signals,

and thus new methods are needed to follow Rho GTPase activity simultaneously with other signaling molecules in cells.

Even though there are 20 Rho GTPases and most of them affect the cytoskeleton in some way, our understanding of how they regulate protrusions is based primarily on Cdc42, Rac1/2, and RhoA. Perhaps this is because these are the mostly highly conserved GTPases in eukaryotes (Boureux et al., 2007), and hence, they are the ones that are actually central to protrusion, whereas the other ones serve more specialized functions in specific cell types.

New microscopy techniques should allow us to visualize in ever-greater detail the interactions and localization of proteins in protrusions. Super-resolution microscopy provides important in-depth snapshots of protein localization, although so far it is not possible to use these methods for rapid live-cell imaging (Toomre and Bewersdorf, 2010). Rapid imaging techniques now being used to visualize cells in vivo will provide us with better insight into how cells coordinate membrane protrusions at the leading edge in physiological and pathological environments.

Cell migration is central to many chronic human diseases, including cancer, cardiovascular disease, and chronic inflammation. Therefore, new insights into the crucial molecules required for cell protrusion will be important in designing therapies to counter these diseases.

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